Natural Occurrence of *Campylobacter* Species, *Salmonella* Serovars, and Other Bacteria in Unabsorbed Yolks of Market-Age Commercial Broilers

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Primary Audience: Production Managers, Microbiologists, Veterinarians, Processing Plant Managers

SUMMARY

In the developing avian embryo, the main energy source is the yolk. Toward the end of the incubation period, the remaining yolk sac is internalized into the abdominal cavity. At hatch, the remaining yolk comprises 20% of the chick's BW and provides the nutrients needed for maintenance. Posthatch, chicks rapidly initiate the transition from yolk dependence to the utilization of exogenous feed. However, at present, it is not known what types of bacteria are found to be associated with unabsorbed yolk sacs from market-age broilers. For Experiment 1, one hundred 6-wk-old defeathered broiler carcasses were obtained from a commercial processing facility during each of 3 visits. In the second experiment, one hundred 8-wk-old defeathered broiler carcasses were obtained from a different commercial processing plant on 4 separate occasions. For both experiments, each carcass was aseptically opened and inspected for the presence of an unabsorbed yolk sac. Three to 5 carcasses containing a free-floating yolk sac (within the abdominal cavity) and the yolk stalk (without a yolk sac) and 3 to 5 carcasses containing an attached yolk and yolk stalk from each repetition were randomly selected and analyzed for levels and types of total aerobic bacteria (APC), *Enterobacteriaceae* (ENT), and for the presence of *Campylobacter* spp. and *Salmonella* serovars. The APC ranged from log 3.3 to >log 6.0, and the ENT ranged from log 2.8 to >log 6.0. *Staphylococcus* spp. and *Streptococcus* spp. were the predominant organisms in APC, whereas *Escherichia coli* and *Hafnia alvei* were found to comprise the ENT. *Campylobacter* spp. was found in 29% of the yolk stalks, 32% of the attached yolk sacs, and 13% of the free-floating yolk sacs. All *Campylobacter* isolates were determined to be *Campylobacter jejuni*, except for 1 attached yolk and yolk stalk, which was *Campylobacter coli*. *Salmonella* serovars were found in 26% of the yolk stalks, 48% of the attached yolk sacs, and 23% of the free-floating yolk sacs, and the majority of *Salmonella* isolates were *Salmonella* Typhimurium. The significance of these bacterial reservoirs and carcass contamination during processing is yet to be determined.

Key words: *Campylobacter*, *Salmonella*, *Enterobacteriaceae*, unabsorbed yolk sac, broiler

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DESCRIPTION OF PROBLEM

The main energy supply of the developing avian embryo is yolk, which contains carbohydrates, lipids, proteins, and antibodies, with lipids providing the most important source of energy to the embryo [1, 2]. Near the end of the incubation period, the yolk sac is internalized into the abdominal cavity. At the time of hatch, the remaining yolk comprises approximately 20% of the chick's BW and continues to provide immediate posthatch energy, protein, and water for maintenance [1, 3]. The yolk sac (saccus vitellinus) is a thick-walled, opaque structure connected to the small intestines by the yolk stalk (ductus vitellinus) and opens on the antimesenteric side at the jejunoileal junction (sometimes called midileum), which lies within the distal half of the small intestines. In most instances, the yolk will be depleted during the first 7 d posthatch, and the yolk sac will be completely absorbed, leaving the yolk stalk, also known as the Meckel's diverticulum, a lymphoepithelial organ [4, 5, 6, 7, 8]. However, it has been observed that numerous broilers in the processing plant contain what is commonly called unabsorbed yolk contents. In a recent study, Buhr et al. [9] found that 49% of the broilers at market-age had an unabsorbed yolk sac, and 34% of the yolk sacs were attached to the yolk stalk and 15% were free-floating within the abdominal cavity.

Campylobacter and *Salmonella* contaminations are a major concern to the poultry industry due to the organisms being recognized as causes of acute bacterial gastroenteritis in humans [10, 11, 12]. *Campylobacter* and *Salmonella* are mainly associated with the alimentary tract of poultry. However, recent findings suggest that *Campylobacter* and *Salmonella* can be readily detected in numerous internal organs and tissues, as well as in the alimentary tract of broilers and broiler breeders [13, 14, 15, 16, 17, 18].

There is little, if any, published information on the natural bacteriology of unabsorbed yolk sacs, particularly concerning market-age broilers. However, there have been papers reporting the presence of *Salmonella* spp. in the remaining yolk of 1-d-old chicks and ostrich chicks [19, 20, 21]. Dzoma and Dorrestien [19] looked at the bacteriology of the unabsorbed yolk sacs of ostrich chicks that ranged from 1 to 21 d of age. Of the 80 yolk sacs they studied, 22% were infected with bacteria, and the same species were generally isolated from the liver (only rod-shaped bacteria were observed). No *Campylobacter* species, spore-forming bacteria, or fungal elements were observed. *Escherichia coli* was the most common isolate found in the unabsorbed yolks. However, they also found *Pseudomonas mesophilia*, *Pseudomonas pneumonia*, *Serratia liquefaciens*, *Alcaligenes xylosopsid*, *Aeromonas hydrophila*, and *Enterobacter cloacae*.

The objectives of this study were as follows: 1) to determine whether *Campylobacter* spp. and *Salmonella* serovars, along with other bacteria, are naturally present in the unabsorbed yolk sacs of market-age commercial broilers, and 2) to determine what bacterial species are common in these unabsorbed yolks.

MATERIALS AND METHODS

Experimental Design

For Experiment 1, one hundred 6-wk-old broiler carcasses were obtained from a commercial processing facility on each of 3 separate visits. For Experiment 2, one hundred 8-wk-old broiler carcasses were obtained from a different commercial processing plant on each of 4 separate visits. For both experiments, the carcasses were removed from the processing line following defeathering (rehang table) and transported to a research pilot processing plant. Each carcass was aseptically opened and inspected for the presence of an unabsorbed yolk sac. The antimesenteric side of the jejunoileal segment of the small intestine was examined for the presence or absence of a yolk stalk called the Meckel's diverticulum. Those with unabsorbed yolk sacs were further separated into 2 groups, attached to the yolk stalk or free-floating within the abdominal cavity.

For Experiment 1, three carcasses containing a free-floating yolk and yolk stalk and 3 carcasses containing an attached yolk were randomly selected from the 100 carcasses from 2 replications, and 5 carcasses each from the last replication were randomly selected from

the 100 carcasses and analyzed for levels and types of total aerobic bacteria (**APC**), *Enterobacteriaceae* (**ENT**), *Salmonella*, and *Campylobacter*.

For Experiment 2, five carcasses containing a free-floating yolk and yolk stalk and 5 carcasses containing an attached yolk for all 4 replications were randomly selected from each of the 100 carcasses and analyzed for levels and types of APC, ENT, *Salmonella*, and *Campylobacter* spp. To reduce the possibility of cross-contamination among samples, the unabsorbed yolk sacs were aseptically removed before collection of the ceca. Individual samples (free-floating yolks, yolk stalks, attached yolk and yolk stalk, and ceca) were placed in sterile bags, packed on ice, and transported to the laboratory for evaluation. The samples within plastic bags were then macerated with a rubber mallet to ensure that the contents of the samples were exposed and standard laboratory procedures for *Campylobacter*, a modified laboratory procedure for recovery of *Salmonella*, and determination of ENT and APC were performed on the samples. Due to the size (<1 g to >15 g) of the sample tissues (unabsorbed yolks), Bolton's broth was added to the samples to better evaluate incidence of *Campylobacter*, along with *Salmonella*, ENT, and APC. This procedure was validated in preliminary studies (data not shown) and was adequate for recovery of the organisms of interest. In addition, for samples that weighed >2 g, a ratio of 1:3 was used; for samples weighing <2 g, a standard of 6 mL was added to the sample bags to provide enough diluent for all enumerations.

Campylobacter Lab Procedure

Standard laboratory methods for the recovery of *Campylobacter* spp. were performed utilizing Bolton's enrichment broth (containing lysed horse blood) and Campy-Cefex agar [13]. All confirmed isolates were frozen on commercially available ceramic beads in cryopreservative fluid [22] and held at −80°C until species identification could be performed.

Campylobacter Speciation

Isolates were obtained from the −80°C freezer and placed onto Campy-Cefex agar and incubated for 48 h at 42°C in a microaerophilic condition (5% O_2, 10% CO_2, and 85% N). The isolates were then picked and placed onto blood agar plates and incubated at 42°C for 24 h. Template DNA was prepared by picking 3 to 4 colonies from a plate using a sterile disposable plastic loop and BAX PCR system [23] utilized for speciation of *Campylobacter jejuni* and *Campylobacter coli*.

Salmonella Lab Procedure

A validated laboratory procedure was utilized, in which a 1-mL aliquot of solution from the above Bolton's enrichment broth after stomaching was added to 9 mL of buffered peptone water [24]. Standard laboratory procedures were then performed utilizing TT Broth (Hajna) and brilliant green S and modified Lys Fe agar for plating media [24]. Confirmed colonies were streaked onto Tryptic soy agar (**TSA**) [25] and incubated overnight at 37°C. Parafilm [26] was then wrapped around the top of each TSA tube and saved at room temperature.

Salmonella Speciation

All saved TSA slants containing *Salmonella* isolates were shipped to the USDA National Veterinary Services Laboratory in Ames, IA for serovar identification.

Aerobic Plate Count Lab Procedure

For Experiment 1 and 2, serial dilutions were made from the unabsorbed yolk sac samples after the addition of Bolton's enrichment broth.

Aerobic populations were enumerated on plate count agar and incubated at 37°C for 24 h. Populations were counted and reported as the base-10 logarithm colony-forming units per gram of tissue sampled. From each plate, 3 to 5 colonies were picked from the plate count agar and streaked for isolation onto nutrient agar, and the VITEK 2 [27] was utilized for determination of colony types.

ENT Lab Procedure

For Experiments 1 and 2, serial dilutions were made from the unabsorbed yolk samples after the addition of Bolton's enrichment broth.

Enterobacteriaceae was enumerated on violet red bile agar with 1% glucose added with an overlay. Plates were incubated at 37°C for 24 h. Presumptive colonies (purple-red colonies) were counted and reported as the base-10 logarithm colony-forming units per gram of tissue sampled. From each plate, 3 to 5 colonies were picked from the violet red bile agar and plated onto nutrient agar. Following incubation, Micro-ID [25] was utilized for determination of types of ENT present.

RESULTS AND DISCUSSION

For Experiments 1 and 2, the APC ranged from log 3.3 cfu/g to >log 6.0 cfu/g for all sample sources. The ENT ranged from log 2.8 cfu/g to >log 6.0 cfu/g. *Staphylococcus* spp. and *Streptococcus* spp. were the predominant organisms in APC. *Staphylococcus* spp. was found to associate with the attached yolk sac samples, whereas *Staphylococcus* spp. was found in only the yolk stalk or Meckel's diverticulum samples. *Escherichia coli* was found to be the predominant organism in ENT, and in certain instances, *Hafnia alvei* was also found. *Escherichia coli* was found to associate with all sample sites (attached yolk sac, yolk stalk, and free-floating yolk sac). *Hafnia alvei* was isolated from only the yolk stalk samples.

Dzoma and Dorrestein [19], when examining the bacteriology of the unabsorbed yolk sacs of ostrich chicks, found only rod-shaped bacteria, and they reported that *E. coli* was the most common isolate found in the unabsorbed yolks. However, they also found numerous other bacteria such as *P. mesophilia*, *P. pneumonia*, *S. liquefaciens*, *A. xylosopsid*, *A. hydrophila*, and *E. cloacae*. In a related study, APC and ENT were found in levels of up to log 3.5 cfu/g in inoculated, experimentally raised 6-wk-old broilers [18]. Therefore, these unabsorbed yolks, whether they are free-floating or attached, can harbor numerous types of bacteria.

For all experiments, *Campylobacter* spp. was found in 29% (10 out of 31) of the yolk stalks, 32% (10 out of 31) of the attached, and 13% (4 out of 31) of the free-floating yolk sacs (Table 1). *Salmonella* serovars were found in 26% (8 out of 31) of the yolk stalks, 48% (16 out of 31) of the attached, and 23% (7 out of 31) of the free-floating yolk sacs. *Campylobacter* and *Salmonella* were simultaneously recovered from a yolk stalk and from 3 ceca from the 6-wk-old carcasses. In the 8-wk-old carcasses, *Campylobacter* and *Salmonella* were simultaneously recovered from 5 unabsorbed yolk sacs, 1 yolk stalk, and 2 ceca.

In Experiment 1 (6-wk-old broilers), *Campylobacter* was isolated from 5 out of 11 attached yolk sacs and from 5 out of 11 corresponding ceca of the attached yolk sampled carcasses. *Campylobacter* was isolated from 3 out of 11 free-floating yolk sacs, 5 out of 11 yolk stalks, and from 5 out of 11 corresponding ceca of the free-floating and yolk stalk sampled carcasses. All isolates from Experiment 1 were determined to be *C. jejuni*. In replication 2, all samples tested were negative for the presence of *Campylobacter* spp. In replication 1, an attached yolk sample was positive, but the corresponding ceca were negative. This also occurred in replication 3, in which a free-floating yolk sac and attached yolk sac were positive, but the corresponding ceca were negative. This phenomenon could have been due to the inability to detect very small numbers of *Campylobacter* spp. with culture methods currently available or due to overgrowth of extraneous bacteria on the plates. In other words, *Campylobacter* spp. may have been in the ceca but was not culturally detected.

In Experiment 1, *Salmonella* serovars were isolated from 3 out of 11 attached yolk sacs and from 1 out of 11 ceca of the attached yolk sac sampled carcasses. *Salmonella* serovars were isolated from 3 out of 11 free-floating yolk sacs, 5 out of 11 yolk stalks, and 1 out of 11 ceca of the free-floating and yolk stalk sampled carcasses. In replication 1, all samples tested were negative for the presence of *Salmonella* serovars. In replication 2, three of the attached yolk samples were positive, but the corresponding ceca were negative. This was also seen in replication 3, in which an unabsorbed yolk was positive and the corresponding ceca were negative, and, as with *Campylobacter*, *Salmonella* may have been in the ceca, but it was not culturally detected. All of the *Salmonella* isolates were *Salmonella* Typhimurium (9).

Table 1. Incidence of *Campylobacter* and *Salmonella* in unabsorbed yolk sacs from commercial broiler carcasses

Item	Sample site	
	Campylobacter[1]	*Salmonella*[1]
Unabsorbed attached yolk sac sampled carcasses[2]		
Unabsorbed yolk sac	10/31	16/31
Ceca	10/31	15/31
Unabsorbed free-floating yolk sac sampled carcasses[2]		
Free-floating yolk sac	4/31	7/31
Yolk stalk	10/31	8/31
Ceca	10/31	14/31

[1]Results for incidence of *Campylobacter* and *Salmonella* are reported as the number of positive samples/number of carcasses sampled.
[2]Combination of repetitions from 6-wk and 8-wk-old commercially obtained broiler carcasses.

In Experiment 2 (8-wk-old broilers), *Campylobacter* spp. was isolated from 5 out of 20 attached yolk sacs and 4 out of 20 ceca from the attached yolk sampled carcasses. *Campylobacter* spp. was isolated from 1 out of 20 free-floating yolk sacs, 4 out of 20 yolk stalks, and 5 out of 20 ceca from the free-floating yolk and stalk sampled carcasses. In replication 2 and 3, all samples tested were negative for the presence of *Campylobacter*. *Campylobacter* again was found in some of the unabsorbed attached yolk samples but not in the ceca. All isolates were determined to be *C. jejuni* except a single attached yolk isolate from replication 4, which was found to be *C. coli*.

In Experiment 2, *Salmonella* serovars were isolated from 12 out of 20 attached yolk sacs and 15 out of 20 ceca from the attached yolk sampled carcasses. *Salmonella* serovars were isolated from 7 out of 20 free-floating yolk sacs, 7 out of 20 yolk stalks, and 12 out of 20 ceca from the free-floating yolk and stalk sampled carcasses. In replication 1, four of the unabsorbed attached yolk samples were positive for *Salmonella*, whereas the corresponding ceca were negative. The majority of *Salmonella* isolates were *S.* Typhimurium (n = 24). However, a few isolates were *Salmonella* Montevideo (9), *Salmonella* Manchester (7), *Salmonella* Kentucky (6), *Salmonella* Thompson (3), *Salmonella* Berta (2), *Salmonella* London (1), and *Salmonella* Schwarzengrund (1).

Campylobacter and *Salmonella* are mainly associated with the alimentary tract of poultry [28, 29, 30]. However, these findings and previous findings suggest that *Campylobacter* and *Salmonella* may be readily detected in numerous internal tissues of broilers in addition to the alimentary tract. The mechanism by which these bacteria reach and colonize these unabsorbed free-floating yolks and internal organs has not been determined. However, in studies by Cox et al. [17] and Bailey et al. [30], it was found that inoculated *Campylobacter* and *Salmonella* could disseminate within 1 h after inoculation to the lymphoid-like organs in 1-d-old chicks that were either inoculated by oral or cloacal routes. This could suggest that the dissemination or presence of *Campylobacter* and *Salmonella* in these internal bodies is due to immune, systemic, or macrophage functions. In addition, *Campylobacter* have been found in the mature and immature follicles of adult broiler breeder hens [15]. However, it was not determined in that study if the liver played a role in follicle contamination.

In the present study, the significance of these unabsorbed yolks as possible reservoirs for these human foodborne enteropathogens and contamination points for broilers being processed is yet to be determined. The yolk stalk of chickens retains its lumen opening into the small intestine by a small papilla. It has been shown that the yolk stalk plays a role in immune function, due to lymphoid cells accumulating and partially occluding the stalk [31, 32]. The lymphoid tissue appears when yolk absorption is nearly complete and regression of the yolk sac quickens. However, yolk stalk closure occurs at different ages, and, in some species, lymphoid invasion does not occur; therefore, it is unknown if or how the yolk stalks close [20,

32]. The yolk stalk may enable the internal cavity of birds to become contaminated with different types of bacteria. If a yolk sac becomes free-floating within the abdominal cavity and the yolk stalk does not completely close, then bacteria originating from the intestines could be excreted into the abdominal cavity of the bird and become peritoneal. Regardless of the cause of contamination of internal bodies, developing an understanding of the viability and presence of *Campylobacter* and *Salmonella* in these internal organs and tissues of broilers could prove to be very beneficial in developing intervention strategies at the farm and processing levels.

CONCLUSIONS AND APPLICATIONS

1. The unabsorbed yolks, whether free-floating or attached, can harbor numerous types of bacteria including *Campylobacter* and *Salmonella* and could be potential contamination points in processing plants.
2. Unabsorbed yolk sacs serve as possible reservoirs for human foodborne enteropathogens and as such may be a source of contamination for other internal tissues and organs.
3. The mechanism by which these bacteria reach and contaminate these unabsorbed free-floating yolk sacs has not yet been determined.

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